The novel link between inflammatory enzyme C2GNT and the shedding of syndecan-1 in podocyte dysfunction

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Abstract

Syndecan-1 is known to be a potential contributor to sub-clinical inflammation in diabetic nephropathy (DN). Loss of syndecan-1 from the surface of podocytes is thought to lead to cell dysfunction, which leads to the detachment of viable podocytes from the glomerulus, an early feature of DN. Although the mechanisms of constitutive syndecan-1 shedding have been addressed by several studies, the pathological mechanisms are less elucidated. The aim of this investigation is to consider the role of the O-glycosylating enzyme C2GNT in syndecan-1 shedding by podocytes. Conditionally immortalised human podocytes were used to study the effect of hyperglycaemia and C2GNT knockdown on syndecan-1 shedding by these cells. Hyperglycaemia induced C2GNT activity in podocytes results in increased O-glycosylation on the surface syndecan-1 in cells treated with high glucose compared to percentage of normal glucose (219.5±145.7 vs 100%, P<0.05). This increase in O-glycosylation is associated with an increase in the shedding of the syndecan-1 ectodomain by podocytes treated with high glucose compared to percentage of normal glucose (118.2±7.1 vs 100%, P<0.05). Moreover, podocytes manipulated for C2GNT knockdown show reduced syndecan-1 shedding when treated with high glucose compared to wild type cells treated with high glucose (89.97±11.95 vs. 118.2±7.17, P<0.05). Our findings suggest that the activity of O-glycosylating enzyme C2GNT is raised in podocytes under diabetic conditions. We demonstrate for the first time a novel mechanism of pathological syndecan-1 shedding induced by C2GNT activity. This excess syndecan-1 shedding by podocytes can contribute to podocyte dysfunction.

Introduction

Diabetic nephropathy (DN) is a complication of the microvasculature of the kidneys. Clinical and epidemiological evidence suggests that up to 20% of patients with Type 1 diabetes (T1DM) and 40% of patients with Type 2 diabetes (T2DM) will develop DN. With the advent of the diabetes pandemic worldwide, the incidence of DN is also on a steep rise.1 DN is closely associated with the development of cardiovascular disease in diabetes and contributes to early mortality as well as the need for renal replacement therapy and dialysis.1,2 This makes the understanding of the pathogenesis of this complication and need for novel therapeutic targets crucial.

Syndecan-1 is known to play an important role in influencing cell polarity, cell matrix interaction, anchorage of the cell to its basement membrane and bearing growth factors to appropriate receptors.3 Loss of syndecan-1 on epithelial cells is known to lead to loss of anchorage dependence and cell dysfunction. This phenomenon has been further studies as epithelium to mesenchyme transition (EMT) and cell migration.4 EMT is a complex process comprising multiple stages characterised by the loss of conventional cell surface markers.13 EMT is also recognised as a survival response whereby cells change morphology as a result of injury, but fail to undergo apoptosis.14,15

Podocytes or glomerular epithelial cells are highly specialised quiescent cells that play a key role in forming the glomerular filtration barrier.15 Podocyturia or the presence of viable podocytes in the urine of patients, is an early pathological feature of DN.16 This pathological feature is considered a strong predictor of the progression of DN and associates closely with proteinuria and glomerulosclerosis.17-20 EMT has been implicated in podocyte dysfunction by several studies.21-23 Core-2-β-1,6-N-acetylglosaminyltransferase (C2GNT) is an inflammatory enzyme that brings about the addition of specific O-glycans to serine/threonine rich proteins thus forming core-2-structures. These O-glycan structures have been recognised for both physiological and pathological contributions in
cell-cell communication and the development of metastasis. Recently, there has been great interest in the role of C2GNT in the inflammatory response. C2GNT brings about changes in adhesion molecules such as P-selectin glycoprotein ligand 1 (PSGL-1) which in turn increases the extent of leukocyte homing and adhesion. The systemic link between diabetic retinopathy and nephropathy, and C2GNT activity has previously been established indicating strong correlations between the activity of the enzyme in leukocytes of the patients and the progression of the pathology. At the cellular level, C2GNT is known to bring about post-translational modifications that influence cell signaling, cell-cell communication and cell-matrix interaction via surface proteins. We propose that under hyperglycaemic conditions, C2GNT activity in podocytes is raised. Raised C2GNT activity leads to an increase in the O-glycosylation on the ectodomain as well as the JM stalk of the transmembrane domain of syndecan-1. This change in O-glycosylation influences shedding of the syndecan-1 ectodomain. The subsequent loss of syndecan-1 by podocytes leads to podocyte dysfunction and detachment. This hypothesis provides a novel mechanism of podocyturia, and a novel potential therapeutic target for DN.

Materials and Methods

Podocyte cell culture and treatment
Conditionally immortalised human podocytes developed and described by Saleem et al. were cultured in RPMI containing 5.6 mM glucose, supplemented with 10% fetal serum (FBS), insulin (1 mg/mL), transferrin (0.55 mg/mL) and selenium (0.5 μg/mL) supplement (Gibco, Paisley, UK) and 1% penicillin and streptomycin. Podocytes were exposed to normal glucose (NG, 5.6 mM glucose), high glucose (HG, 35 mM glucose) or to mannitol which served as an osmotic control (M, 35mM mannitol) for 24 hours. All cells were treated after 14 days of incubation at non-permissive temperature to ensure differentiation.

Caspase-3 activity assay
A caspase-3 activity assay kit (Sigma Aldrich, Dorset, UK) was used to measure caspase-3 activity in treated cells as an indication of cell death and apoptosis. This fluorometric kit measures endogenous caspase-3 activity of a sample depending on its capacity to hydrolyse the substrate acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin, Ac-DEVD-AMC. The resulting fluorescence is measured at an excitation wavelength of 360 nm and emission wavelength of 460 nm. Caspase-3 activity is calculated on the basis of a calibrated curve generated by using AMC standards. Final activity is normalised against protein concentration. Each experiment was repeated on five separate occasions and the mean taken.

Ethydim homodimer staining
Another method to test cell viability based on the integrity of the cell membrane is by staining with ethidium homodimer (Molecular Probes, Paisley, UK). This stain cannot enter viable cells, however in dead cells it readily permeates and binds to nucleic acid. The fluorescence is highly pronounced once the stain binds DNA and can be measured at an excitation wavelength of 528 nm and emission wavelength of 517 nm. The experiment was repeated on three separate occasions (in triplicates on each occasion) and the mean taken.

Intracellular glucose concentration-Amplex red assay
Intracellular glucose concentration was measured using a commercially available kit, Amplex Red® Glucose assay kit (Invitrogen, Paisley, UK). The protocol and principle were provided by the manufacturers. To 50 mL of diluted cell lysates 50 μL of reaction mixture containing 10 mM Amplex Red®, 10 U/mL horse radish peroxidase (HRP), 100 μM of glucose oxidase and 50 mM of sodium phosphate buffer were added. This mixture was incubated at room temperature for 45 minutes under dark conditions. At the end of the incubation period the fluorescence was measured with a fluorometer (Spectra Max M2, Molecular Devices, Berkshire, UK) at an excitation wavelength of 530-560 nm and emission of 590 nm. The concentration of intracellular glucose was calculated based on a standard curve generated as per supplier instructions. The calculated concentration of each sample was normalised using the protein concentration of the samples and the final results expressed as μM of glucose/μg of protein. The experiment was repeated on ten separate occasions and the mean taken.

General cellular reactive oxygen species measurement
Cellular reactive oxygen species (ROS) was measured using a fluorogenic probe 2’,7’- dichlorofluorescein diacetate (H2DCFDA) (Molecular Probes, Paisley, UK). DCFDA readily enters cells and measures hydroxyl, peroxyl and other ROS. The probe is first deacetylated by esterases in the cytosol and this product is oxidized by ROS to a highly fluorescent entity, 2’, 7’ - dichlorofluorescin. This fluorescence in measured using a fluorometer (Spectra Max M2, UK) with excitation at 495 nm and emission at 529 nm. The experiment was repeated on four separate occasions (in triplicates on each occasion) and the mean taken.

Mitochondrial reactive oxygen species measurement
Mitochondrial ROS was measured using a non-fluorescent probe Dihydrorhodamine 123 (DHR 123) (Molecular Probes, Paisley, UK). DHR 123 is uncharged and therefore can diffuse across membranes where it is oxidized to negatively charged rhodamine 123. This oxidation product localises in the mitochondria and displays a fluorescence that can be detected using a fluorometer (Spectra Max M2, Molecular Devices, Berkshire, UK) with excitation at 500 nm and emission at 536 nm. The experiment was repeated on four separate occasions (in triplicates on each occasion) and the mean taken.

Measurement of core-2-β-1,6-N-acetylglucosaminyltransferase activity
The assay was performed as previously described. Final concentrations of 40 mM 2-((N-morpholino) ethanesulfonic acid (MES), pH 7.0, 0.5 μCi of UDP-[1H]GlcNAc (Perkin Elmer, Cambrigeshire, UK) in 0.8 mM UDP-GlcNAc (Sigma Aldrich, Poole, UK), 80 mM GlcNAc (Sigma Aldrich, Poole, UK) and 15 μM of lysates were mixed with acceptor 0.8 mM p-nitrophenyl-gal-3GalNAc (Toronto Research Chemicals, Canada), for a positive reaction or without acceptor for negative reaction to give the endogenous activity of the enzyme. This reaction mixture was incubated at 37°C for 1 hour. At the end of the incubation period the reaction mixture was purified using column chromatography with C18 Sep-Pak columns (Agilent Technologies, Berkshire, UK) on a vacuum manifold (Sigma Aldrich, Poole, UK). The purified product was eluted using absolute methanol into scintillation cocktail (Perkin Elmer, Cambrigeshire, UK). The final elution was used to measure scintillation using a beta liquid scintillation counter (LS 6500, Beckman Coulter, High Wycombe, UK). The enzyme activity was expressed as pmol/h/mg of cell protein. The experiment was repeated on six separate occasions, and the mean taken.

Measurement of shed syndecan-1 in conditioned media
An enzyme-linked immunosorbent assay (ELISA) was used for the quantification of plasma Syndecan-1 (CD 138 ELISA Diaclone, distributed in the UK by 2B Sciences, Oxfordshire, UK). The kit is based on a sandwich ELISA. Monoclonal anti-CD138 antibody pre-coated microtiter strip plates was supplied by the manufacturer. Plasma samples and biotinylated anti-CD138 were added to these coated wells and incubated together at room temperature for 1 hour. At the end of this incubation period the wells were washed thorough-
ly to remove excess and non-specific binding. This was followed by a 30 minute incubation with HRP conjugated streptavidin at room temperature. At the end of this incubation, the wells were washed thoroughly and 100 μL of HRP substrate (TMB) added to all wells and allowed to develop for 12-15 minutes. This reaction was stopped by the addition of 100 μL sulphuric acid, and the absorbance read at 450 nm wavelength. The concentration of shed syndecan-1 in given samples was calculated based on a standard curve generated using the standard provided by the manufacturers. The experiment was repeated on six separate occasions.

**Heparanase enzyme activity assay**
The activity of heparanase enzyme (HPSE) was measured in samples using an enzyme immunoassay (Heparan degrading Enzyme EIA Kit, Takara, Japan). The experiment was repeated on five separate occasions.

**Isolation of membrane and cytosolic fractions**
The membrane and cytosolic fractions were prepared based on the use of two separate lysis buffers and two different centrifugation speeds. The method developed and described by Evans et al. was used for this purpose.33

**Immunoprecipitation and western blot**
An antibody raised against Syndecan-1 (Biolegend, Cambridge, UK) was coupled with Pan mouse IgG Dynabeads® (Invitrogen, Paisley, UK) and used to immunoprecipitate syndecan-1 from conditioned media overnight at 4°C. A monoclonal antibody raised against o-glycan structures (Abcam, Cambridge, UK) was used for the Western blot analysis. Anti-syndecan-1 antibody was used as a loading control. The experiment was repeated on five separate occasions.

**Figure 1.** High glucose does not induce cell death in podocytes. Confluent differentiated podocytes were exposed to normal glucose (NG, 5.6 mM), high glucose (HG, 35 mM) and mannitol (M) of the same concentration as an osmotic control (M, 35 mM) for a period of 24 hours. After the treatment, the cells were washed twice with PBS. The treated cells were either lysed for the measurement of caspase-3 activity (A) or ethidium homodimer, (B) was added to cells grown in 96 well plates. Data are presented as mean±standard deviation of 5 separate experiments. *P<0.05 vs. HG. C), D) and E) represent undifferentiated podocytes, differentiated podocytes under normal glucose conditions and viable reattached cells after hyperglycaemic treatment for 24 hours.

**Figure 2.** Exposure to high glucose increases intracellular concentration of glucose in podocytes. Confluent differentiated podocytes were exposed to NG, HG and M for a period of 24 hours. After the treatment, the cells were washed twice with PBS and lysed for the measurement of intracellular glucose using Amplex® Red glucose assay kit (Molecular Probe). Data are presented as mean±standard deviation of 10 separate experiments. *P<0.05 vs. NG.

**Figure 3.** High glucose-induced cellular and mitochondrial ROS. Confluent differentiated podocytes were exposed to NG, HG and M for a period of 24 hours. After the treatment, the cells were washed twice with PBS and (A) probes DHR 123 and (B) DCFDA were incubated with the cells for 1 hour. Data are presented as mean±S.D. of 4 separate experiments. *P<0.05 vs. NG.
Transfection of podocytes

Podocytes were grown and differentiated in 6-well plates. To 1.5 mL sterile microfuge tube, 250 mL of serum free media was added followed by 6 mL of TransIT-siQuest\textsuperscript{®} transfection reagent (Mirus, UK), the contents were mixed briefly. To the microfuge tube 25 nM of C2GNT siRNA was added and thoroughly mixed and then incubated at room temperature for 30 min. The prepared transfection reagent with siRNA C2GNT (Santa Cruz, UK) was added drop by drop to approximately five different areas of the 6-well plate. The dish was gently shaken to ensure thorough mixing and incubated for 48 hours for further treatment.

Protein measurement

Total protein was measured using the bicinchoninic acid (BCA) protein assay kit (Sigma, Poole, UK).

Statistical analysis

The statistical analysis was performed using GraphPad Prism. Statistical comparisons between groups were studied using student's t-test. A probability level of P<0.05 was chosen as the threshold for acceptance of statistical significance. All data are represented as mean±standard deviation.

Results

Cell death/toxicity

Caspase-3 activity was neither raised nor significantly lowered in podocytes exposed to high glucose (HG) (89.36%±32.6) compared to percentage of normal glucose (NG) (100%) and mannitol (M) (108.4±50.3%), thus suggesting that a 24 hours HG exposure does not lead to apoptosis in podocytes in vitro (Figure 1A). This has previously been observed in a neuronal cell line.\textsuperscript{34} Similar results were observed when cell death on the basis of membrane integrity (ethidium homodimer) was considered (Figure 1B). HG and M (74.6±23.9 percentage of NG and 97.7±36.9 percentage of NG) were not significantly different from NG 100%). It was also observed that podocytes that were detached at the end of a 24 hours HG treatment reattached to culture dishes when replaced in NG (Figure 1E). These cells appear to be morphologically similar to undifferentiated podocytes (Figure 1C) and dissimilar to fully differentiated podocytes in NG (Figure 1D).

Intracellular glucose

Intracellular glucose concentration was measured and expressed as glucose \( \mu M/\mu g \) protein. The intracellular concentration of glucose in podocytes exposed to HG was significantly higher (606.3±485.1 \( \mu M/\mu g \) protein) compared to NG (83.4±50.3 \( \mu M/\mu g \) protein). This was confirmed by Western blot of the podocyte lysate using antibodies against glucose transporter 1 (GLUT1). A significant increase in GLUT1 expression was observed in HG and M compared to NG (Figure 2).

Figure 4. High glucose treatment raises C2GNT activity in podocytes. Confluent differentiated podocytes were exposed to NG, HG and M for a period of 24 hours. After the treatment, the cells were washed twice with PBS. The treated cells were lysed for the measurement of C2GNT activity. Data are presented as mean±S.D. of 6 separate experiments. * P<0.05 vs. NG.

Figure 5. High glucose induced increase in Syndecan-1 shedding in the conditioned media of podocytes. Confluent differentiated podocytes were exposed to NG, HG and M for a period of 24 hours. After the treatment, conditioned media was collected and centrifuged at 2000 rpm for 5 minutes to ensure the removal of cells and cellular debris. This conditioned media was then used for the measurement of shed syndecan-1. Data are presented as mean±S.D. of 10 separate experiments. * P<0.05 vs. NG.

Figure 6. Heparanase (HPSE) activity in podocytes under hyperglycaemic conditions does not change. Confluent differentiated podocytes were exposed to NG, HG and M for a period of 24 hours. After the treatment, the cells were washed twice with PBS. The treated cells were either lysed for the measurement of HPSE activity. Data are presented as mean±S.D. of 5 separate experiments. * P<0.05 vs. NG.
pared to those exposed to NG (110.1±85.3 μM/μg protein) and M (119.3±106.4 μM/μg protein) as depicted in Figure 2.

**Cellular and mitochondrial reactive oxygen species**
Both cellular as well as mitochondrial ROS appear to be raised in podocytes exposed to HG. Mitochondrial ROS levels was significantly raised in HG (38.98±11.95 fluorescence/μg protein) compared to those exposed to NG (25.69±1.13 fluorescence/μg protein) and M (3.01±0.49 fluorescence/μg protein). Cellular ROS levels followed the same pattern with HG vs. NG (20.85±2.86 fluorescence/μg protein vs. 26.11±2.78 fluorescence/μg protein, P<0.05) and M (24.70±11.85 fluorescence/μg protein) (Figure 3A, B).

**Core-2-β-1,6-N-acetylglucosaminyltransferase activity in podocytes**
The activity of the enzyme C2GNT was found to be significantly raised in podocytes exposed to HG compared to those exposed to NG (1.085±842.2 pmol/hr/mg of protein vs. 257.4±274.9 pmol/hr/mg of protein, P<0.05) and M (185±218.6 pmol/hr/mg of protein) (Figure 4).

**High glucose-induced shedding of syndecan-1**
Syndecan-1 levels were significantly higher in podocytes treated with HG compared to those exposed to NG (118.2±7.1 vs. 100, P<0.05) and M (100.5±22.2, P<0.05) as shown in Figure 5.

**Heparanase activity in treated podocytes**
HPSE activity did not change under hyperglycaemic conditions (Figure 6). HPSE activity in HG (10.87±1.1 U/mL) was not statistically different from NG (10.22±0.9 U/mL) and M (10.12±1.3 U/mL).

**Increased O-glycosylation on cytoplasmic domain of syndecan-1**
Figure 7 demonstrates a significant increase in O-glycosylation on the JM region of the transmembrane domain of membrane bound syndecan-1. The level of glycosylation on syndecan-1 molecule on the basis of densitometric ratios was significantly higher in HG compared to NG (219.5±145.7 vs. 100, P<0.05) and M (149.5±58.6).

**Effect of siRNA knockout of core-2-β-1,6-N-acetylglucosaminyltransferase in podocytes on syndecan-1 shedding**
In order to assess the impact of C2GNT on syndecan-1 shedding by podocytes, the differentiated cells transfected with siRNA specific for C2GNT were treated under NG, HG and M conditions. Figure 8 demonstrates that transfected cells treated with HG show less shed syndecan-1 compared to HG in the wild type cell line (89.97±11.95 vs. 118.2±7.17, P<0.05). Transfected cells treated with NG did not result in a significantly different level of syndecan-1 shedding from wild type NG treatment (100.0±2.52). This result provides evidence that C2GNT directly influences syndecan-1 shedding.

**Discussion**
DN poses a great threat to healthcare systems Worldwide as the leading cause of end-stage renal diseases (ESRD) and the most common cause for renal replacement therapy.1,35-37 Clinical evidence suggests that approximately 15-20% of patients with Type 1 diabetes and 30-40% of patients with Type 2 diabetes will develop ESRD.1,38 DN is also closely associated with increased cardiovascular morbidity and mortality.39 Several factors and pathways con-
tubular cells.\textsuperscript{50} We propose that increased O-glycosylation on the juxtamembrane domain promotes shedding by proteases in angiotensin converting enzyme 2 (ACE) activity and syndecan-1 ectodomain shedding via an increase in C2GNT activation.\textsuperscript{53}

Syndecan-1 and C2GNT are both known to play an important role in cell-cell adhesion and cell-matrix adhesion.\textsuperscript{5} The loss of syndecan-1 on podocytes triggers EMT and the cells become anchorage independent. We have also observed that these cells show a failure to undergo apoptosis. A 24 hours hyperglycaemic treatment results in the detachment of some podocytes, but when these detached cells are plated in media containing normal glucose concentration, the cells reattach to the culture dish and display an altered morphology. This is consistent with clinical studies that have observed the loss of viable podocytes in the urine of patients with DN.\textsuperscript{27} Furthermore, Figure 8 indicates for the first time, that C2GNT knockdown in podocytes and subsequent loss of C2GNT activity in these cells results in a decrease in syndecan-1 shedding by these cells under hyperglycaemic conditions. This work provides evidence for the first time of a direct link between C2GNT activity and syndecan-1 shedding. Figure 9 summarises the role of C2GNT in the shedding of syndecan-1 ectodomain and subsequently the progression of DN.

Figure 9. C2GNT and Syndecan-1: Link in DN. This flowchart depicts the hypothesis that we have investigated. We suggest that hyperglycaemia induced C2GNT activation results in increased O-glycosylation on syndecan-1. This in turn influences the shedding of the syndecan-1 ectodomain. The shed ectodomain contributes to inflammation systemically as it enters the vasculature, this in turn contributes to the progression of DN. Locally in the kidneys, the loss of syndecan-1 from podocytes leads to EMT, which in turn results in detachment of podocytes from their basement membrane; a characteristic early feature of DN.

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contribute to DN as a result of its chemokine signalling locally in the glomerulus. We have previously shown in a clinical study that the activity of the inflammatory enzyme C2GNT is closely associated with the progression of DN. Therefore we conclude from this study that the enzyme C2GNT may contribute to DN both systemically via PMBCs and locally in podocytes via increased shedding of syndecan-1. Future work would include the use of C2GNT inhibitor to test the change in loss of syndecan-1 by podocytes, this would allow us to recognise C2GNT as a potential therapeutic target for DN.

References